Development of a sequential injection system for the determination of nitrite and nitrate in waters with different salinity: Application to estuaries in NW Portugal

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In this work, a sequential injection methodology for monitoring nitrite and nitrate in estuarine waters without any previous treatment is described. The developed system was applied to the measurement of nitrite and nitrate in estuarine waters of three rivers in the NW Portugal, allowing an automatic, fast (ca 60 h^{-1}) and precise method (relative standard deviation lower than 2%). The procedure was based on the reaction between nitrite, sulfanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride (N1NED), whereas the determination of nitrate resulted from its reduction to nitrite, using an in-line cadmium column, followed by the same reaction. The samples were collected in three locations for each river (Douro, Cávado and Ave) covering the lower, middle and upper section of the estuaries. Despite the presence of a salinity gradient, this parameter showed no interference in the accuracy of the determinations. The results obtained for the described method for nitrite were statistically comparable to those obtained by the reference procedure. For the determination of nitrate, recovery tests confirmed that the sequential injection methodology provided good quality results.

1. Introduction

Although nitrogen is among the macronutrients required for metabolism and growth of organisms, it cannot be taken up directly by eukaryotic organisms from the molecular form present in the atmosphere, where it is the most abundant species (about 80%). Nitrogen must first be bound and converted to nitrate (nitrification process). For this is reason, nitrogen fertilizers based on nitrate are so commonly used. The extensive use of these fertilizers leads to an increase of nitrogen (in the form of nitrate) in the environment as crops take up a relatively small part of all the fertilizers used, between 25 and 30%. This excess of nitrogen leaches into groundwater and surface water through soils, due to nitrate solubility. Together with nitrite and ammonium, they represent the most common ionic forms of dissolved inorganic nitrogen in aquatic ecosystems.[1] Considering that ammonium and nitrite tend to be oxidised to nitrate by aerobic prokaryotic microorganisms (bacteria and archaea), they all contribute to the increase of nitrate concentration. Over the past decades, human activity has led to a significant alteration in the nitrogen cycle which has increased both the availability and mobility of nitrogen in the environment.[1] The health consequences of high human ingestion of inorganic nitrogen such as nitrate may result in serious illness due to its conversion to nitrite in the body and consequent reduction of the blood capacity to carry oxygen, a condition that can ultimately lead to death.[2] This concern was already translated by the European Union Nitrates Directive[3] which requires that appropriate controls on agricultural inputs of nitrate must be put in place.

The increase of nitrate in water bodies such as river, lakes, estuaries and the coastal zone, can lead to eutrophication, meaning excess of nutrients followed by algal blooms, oxygen depletion and fish deaths. An estuary being by definition an area where "sea water is measurably diluted with freshwater derived from land drainage",[4] serves as a boundary system between the fluvial environment and the coastal zone. Indeed, estuaries may exhibit typical environmental gradients with its own habitats and species making them an irreplaceable natural resource.

The monitoring of nitrites and nitrate in estuarine waters is an effective way to trace possible contamination sources. But estuarine waters are rather complex matrices as they change significantly with the proximity or distance from the sea end, both in terms of concentration values for many analytes and physicochemical properties such as temperature, pH and conductivity.

Sequential injection analysis has proved to be an effective tool for meeting the requirements of routine water analysis[5] adding robustness and versatility to the other flow methodologies characteristics of automation, high throughput and reagent economy. The application of SI methodologies to the determination of inorganic nitrogen ionic forms has been quite successful. Most authors describe the spectrophotometric determination of nitrite based on a colorimetric reaction and then present either the concentration of nitrite,[6–8] or the concentration of both nitrate and nitrite after reduction of nitrate.[9,10]

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Another spectrophotometric approach was described by Mikuska,[11] replacing the traditional colorimetric reaction for chemiluminescent detection. There were also a few works using electrochemical detection systems, such as potentiometry[12] and voltammetry.[13]

Although most of the above mentioned works were applied to waters, only three were applied to saline waters, one applied to coastal waters[6] and two applied to sea waters.[8,10] There is no reference to any work applied to estuarine water samples.

In this research, a sequential injection methodology was developed for the determination of both forms of anionic nitrogen, nitrite and nitrate. This method is based on the Griess reaction between nitrite, sulfanilamide and N1NED which results in a coloured product with maximum absorbance at 543 nm.[14] Therefore, the concentration of nitrite is obtained directly from its reaction with the colour reagent. As for nitrate, it is reduced to nitrite in a copperised cadmium column before the colorimetric reaction; the obtained result corresponds to the sum of nitrite and nitrate in the sample. The nitrate concentration in the sample is then obtained by the difference between the value obtained for nitrite plus reduced nitrate and the one obtained for nitrite.

The described methodology presents the novelty of using two aligned flow cells in the cell compartment (with different optical paths) in order to meet the requirements for the determination of each analyte. In fact, nitrite concentrations in estuarine waters are much lower than nitrate values, and with a narrower concentration range. So, in order to maximize sensitivity for nitrite, a 2 cm optical path flow cell was used. In order to obtain a wider concentration range for nitrate, and not requiring such a low limit of detection, a 1 cm optical path flow cell was used. With this arrangement, the two methodologies could be comprised in the same manifold and use the same spectrophotometer.

The overall conditions of the multiparametric methodology were studied to be applicable to estuarine waters. To assess if the methodology could cope with salinity gradients and other possible interfering agents, we analysed waters from estuaries of three rivers in NW Portugal with different constrains. River Ave watershed (1,400 km^2) drains mainly agriculture and industrial areas, River Cávado (1,600 km^2) crosses through disperse rural and urban areas and River Douro, which drains the largest watershed (98,000 km^2) and presents a much higher freshwater discharge (>500 m^3s^{-1}), flowing into an estuary that suffers the pressure of over one million inhabitants. As a result, the samples collected from these rivers are thought to present different matrices.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and boiled deionised water.

A stock solution of *ortho*-phosphoric acid 5 mol L^{-1} was obtained from the concentrated acid ($d = 1.71$, 85%).

The colour reagent was monthly prepared by dissolving 5 g of sulfanilamide in 25 mL of 5 mol L^{-1} *ortho*-phosphoric acid and by mixing with 0.5 g of *N*-(1-naphthyl)-ethylenediamine dihydrochloride (N1NED) dissolved in water; after homogenizing the mixture the volume was completed to 250 mL. A concentration of 20 g L^{-1} sulfanilamide and 2 g L^{-1} N1NED in 0.5 M of *ortho*-phosphoric acid was obtained in these conditions.

The conditioner of the cadmium column was monthly prepared by dissolving 0.2 g of di-sodium ethylenediamine tetraacetic acid (EDTA) and 10 g of ammonium chloride in 500 mL of water, to a final concentration of 0.4 g L^{-1} EDTA and 20 g L^{-1} ammonium chloride. The pH of this solution was adjusted between 9–9.5 with commercial ammonia solution ($d = 0.91$, 25%).

A standard stock solution of sodium nitrite of 100 mmol L^{-1} was obtained by dissolving 700 mg of the dried solid in water to a final volume of 100 mL. This stock solution was diluted 100 times (1 mmol L^{-1}) (monthly prepared) and from this solution, another dilution (1 : 50) was carried out and this solution, prepared weekly, was 20 μmol L^{-1}.

A standard stock solution of sodium nitrate of 100 mmol L^{-1} was obtained by dissolving 850 mg of the dried solid in 100 mL. From this stock solution, a dilution of 1 : 100 was carried out and this solution (1 mmol L^{-1}) was used for a month.

From the nitrite standard solution of 20 μmol L^{-1} and the nitrate standard solution of 1 mmol L^{-1}, working standards were weekly prepared in the range of 0.50–8.00 μmol L^{-1} of nitrite and 12.5–300 μmol L^{-1} nitrate.

2.2. Cadmium column preparation

The cadmium granules (0.3–1.6 mm, mesh 12–60) were prepared according to the 4500 NO_3^- E method of the Standard Methods for the Examination of Water and Wastewater.[14]

The column tube, made of PTFE with 90 mm long and 3.2 mm inner diameter, was filled with the prepared granules. Ordinary domestic sponge was placed at both ends of the column to entrap the cadmium granules.

After preparing the column, it was washed with conditioner (NH_4Cl-EDTA) and activated by passing a mixture of 25% nitrate solution and 75% conditioner. This mixture resulted from adding 25 mL of a 20 μmol L^{-1} of nitrate (obtained by proper dilution of the 1 mmol L^{-1} solution) to 75 mL of the conditioner solution.

2.3. Sample collection and preparation

As previously mentioned, the estuarine water samples were collected from three different Portuguese estuaries located in NW Portugal: Ave (41.3°N, 08.7°W), Cávado (41.5°N, 08.7°W) and Douro (41.1°N, 08.6°W) rivers.

For each river the water was collected from three different locations, one close to the ocean (location 1) and two others upstream (locations 2 and 3), the number 3 being the location with less influence from the ocean.

The estuarine water samples were introduced in the system either directly or after filtration. For the filtration, a hydrophilic membrane (Whatman, cellulose acetate) of pore size 0.45 μm was used.

2.4. Sequential injection manifold and procedure

The sequential injection manifold used for the colorimetric determination of nitrite and nitrate in estuarine waters is depicted in Fig. 1.

Solutions were propelled by a Gilson Minipuls 3 peristaltic pump with a PVC pumping tube. The pump was connected to the central channel of an eight port selection valve (Valco VICI 51652-E8). All tubing connecting the different components was made of Teflon from Omnifit, with 0.8 mm i.d.

A ThermoSpectromic Helios γ UV-Vis spectrophotometer set at the wavelength of 543 nm used as detection system. For the nitrite determination, the flow-cell was a Starna Brand 75.3 Q (20 mm light path, 40 μL inner volume), and for nitrate determination a Hellma 178.711-QS flow-cell (10 mm light path, 30 μL inner volume). The two flow cells were aligned in the same cell support of the spectrophotometer.

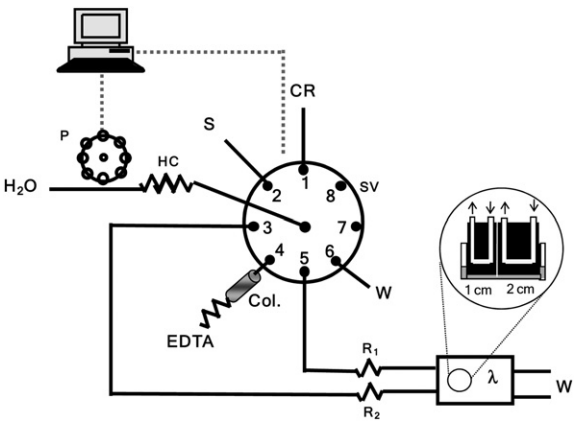

Fig. 1 Manifold for the spectrophotometric determination of nitrite and nitrate in estuarine waters: CR, colour reagent: sulfanilamide 20 g L^{-1} and N1NED 2 g L^{-1} in 0.5 M H_3PO_4; S, water sample or standard; Col., copperised cadmium column of 90 mm long and 3.2 mm width; EDTA, conditioner solution: EDTA 0.4 g L^{-1} and 20 g L^{-1} NH_4Cl; W, waste; P, peristaltic pump; HC, holding coil 400 cm long; SV, eight port selection valve; R_i, reaction coils of 140 cm; λ, spectrophotometer (543 nm) with the two aligned cells.

Analytical signals were recorded in a Metrohm E 586 Labograph strip recorder. A personal computer (Samsung SD 700) equipped with a PCL818L interface card, running with a home-made software written in QuickBasic 4.5, controlled the selection valve (SV) position and the pump sense and speed.

The sequence of steps with the respective time and volumes for the determination of nitrite and nitrate is shown in Table 1.

The first step (step A) is the aspiration of conditioner, through the cadmium column, followed by the aspiration of sample (step B). This way the sample is dispersed in the conditioner. The mixing is promoted by reversing the flow while sending the stacked plugs through the column, for nitrate reduction (step C).

After washing the holding coil, the determination of nitrite is carried out during the nitrate reduction process (steps D to G). For the nitrite determination, the sample is aspirated after the colour reagent, and then the flow is reversed, promoting the mixture on the way to the detector.

After the determination of nitrite and the preparation of the port of the cadmium column (steps H and I), the determination of nitrite + nitrate is carried out (steps J to M). The colour reagent is aspirated, followed by the sample from the column. Then this mixture is sent to the detector.

The coloured product formed corresponds to the sum of the nitrite and the nitrate in the sample. The concentration of nitrate is obtained by the difference between both determinations. The last two steps (N and O) of the protocol sequence aim to prepare the column for a new cycle.

3. Results and discussion

The aspiration order was set in advance being first aspirated the colour reagent followed by sample/standard. This setting was

Table 1 Protocol sequence for the determination of nitrite and nitrate in estuarine water samples

Step	SV position	Time/s	Pump speed	Pump direction	Volume/μL	Description
A	4	4.5	30	a	214	Aspiration of conditioner (EDTA)
B	2	2.5	30	a	119	Aspiration of sample/standard
C	4	10	10	b	148	Propelling to column for reduction
D	8	4	40	b	243	Propelling to waste to wash holding coil
E	1	9	40	a	548	Aspiration of colour reagent
F	2	9.2	40	a	560	Aspiration of sample/standard
G	3	55	40	b	3348	Propelling to detector, mixture of reagent and sample and measurement (nitrite determination)
H	4	3	10	a	44	Aspiration for preparation of the port
I	8	2	40	b	122	Propelling to waste to wash holding coil
J	1	5	40	a	304	Aspiration of colour reagent
L	4	3.4	20	a	102	Aspiration of reduced sample/standard
M	5	45	40	b	2738	Propelling to detector, mixture of reagent and sample and measurement (nitrite + nitrate determination)
N	4	5	30	a	238	Aspiration of conditioner (EDTA) to wash the column
O	8	5	40	b	304	Propelling to waste to wash holding coil

used for both analytical cycles. Also set in advance, the reaction coil length was chosen as the minimal value for connecting the selection valve to the flow cell in the spectrophotometer. Subsequently, some parameters of the developed sequential injection methodology for both determinations were studied, in order to achieve the best sensitivity. These studies were carried out in three stages: firstly the nitrite determination, then the nitrate determination (including the reduction process) and finally the determination of both anions. Some aspects of the colour reagent preparation were also included in the study of nitrite determination, namely the minimization of the *ortho*-phosphoric acid concentration.

3.1. Nitrite determination

For the studies of the nitrite determination, a flow cell of 1 cm light path was used. The first sequential injection parameter to be studied was the aspiration volume (Fig. 2) of the sample. The tested volumes ranged from 285 to 660 μL, and as the sensitivity increased up to 595 μL, this was the sample volume chosen. For the reagent volume, the studied range was 450 to 645 μL and a volume of 580 μL was chosen because it provided the highest sensitivity.

The initial preparation of the colour reagent was done accordingly to the reference procedure,[14] Reagent 1 in Table 2. The colour reagent was composed by different components: sulfanilamide, N1NED and phosphoric acid, the first two being directly involved in the colorimetric reaction.

So, the study of the concentration was carried out maintaining the proportion between sulfanilamide and N1NED of the reference procedure,[14] Reagent 2 and 3 in Table 2.

As the Reagent 2 showed, not only a better sensitivity, but also a better linearity, those were the concentrations chosen for sulfanilamide and N1NED.

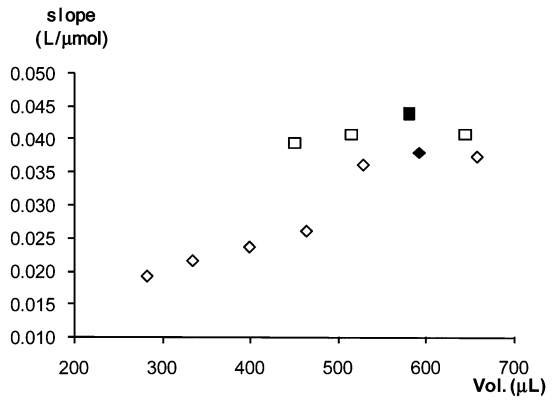

Fig. 2 Study of the aspiration volumes of the sample (◇) and of the reagent (□); the points in black represent the chosen values.

The *ortho*-phosphoric acid not only ensures an acidic pH for the reaction but also contributes to dissolve the sulfanilamide; so, in order to study its minimisation, a batch procedure was used. To assess the minimal quantity of H_3PO_4 required for the dissolution, 0.5 g of sulfanilamide were weighed (for a final volume of 25 mL of colour reagent) and increments of 500 μL of acid were made until complete dissolution. In the end, 2.5 mL of acid were added resulting in an acid concentration of 0.5 M. A calibration curve was made to ensure that the sensitivity had not been affected. Actually, no significant change was observed, the slope had even increased a little ($A = 0.0350\ [NO_2^-] + 0.0181$). Aiming to further increase the sensitivity and to lower the quantification limit, the flow cell of 1 cm light path was replaced by a flow cell of 2 cm light path and, as it could be expected, the sensitivity doubled.

Before incorporating the nitrate determination in the developed system, water samples were analysed, in order to evaluate if there was salinity interference in these conditions. The water samples were analysed by the developed sequential injection methodology and the results compared to the reference procedure.[14]

To evaluate accuracy, a linear relationship between the concentrations obtained with the developed sequential injection methodology (C_{SIA}) and with the reference procedure ($C_{Ref.Met.}$) was established. The equation found was: $C_{SIA} = 1.05\ (\pm 0.021) \times C_{Ref.Met.} - 3.5 \times 10^{-2}\ (\pm 0.052)$; $R^2 = 9.99 \times 10^{-1}$ where the values in parenthesis represent 95% confidence limits. These figures showed that the estimated slope and intercept do not differ statistically from values 1 and 0 respectively. Therefore, there was no evidence for systematic differences between the two set of results.[15]

The linear regression was assessed by residuals analysis. Independence, randomness and normality was validated. The quality of the regression was also confirmed by the coefficient of determination above 0.999.

3.2. Nitrate determination

In order to perform the simultaneous determination of nitrite and nitrate with the same reagents, a reduction process had to be included, to reduce nitrate to nitrite. The chosen procedure was the reduction using solid cadmium, in which nitrate is converted quantitatively to nitrite in the presence of cadmium treated with copper sulfate. The size of cadmium column was set to 90 mm long. Smaller columns provided a very low conversion percentage; a further increase would imply extra steps of washing. The percentage of conversion was calculated by measuring the peak height (H) obtained with a nitrate standard with the H obtained introducing a standard of nitrite with the

Table 2 Study of different concentrations of the colour reagent

Reagent	[Sulfanilamide]/g L^{-1}	[N1NED]/g L^{-1}	[H_3PO_4]/M	Calibration curve
1	10.0	1.0	1.5	$A = 0.0281\ [NO_2^-] + 0.0461$; $R^2 = 0.9933$
2	20.0	2.0	1.5	$A = 0.0307\ [NO_2^-] + 0.0732$; $R^2 = 0.9999$
3	30.0	3.0	1.5	$A = 0.0282\ [NO_2^-] + 0.1012$; $R^2 = 0.9752$

same molar concentration. With the established column dimensions, the conversion percentage was close to 90%. Aiming to increase this value, an option was made to increase the contact time between sample and the copperised cadmium. A 100% conversion was attained by stopping the sample plug in the cadmium column for one minute. This stopping step improved not only the conversion percentage but also the repeatability.

After these studies, two calibration curves were traced, one with nitrite standards and one with nitrate standards with the same concentrations. The slopes were quite similar (slope difference < 2%) as well as the intercepts (difference < 7%). An average of 100.4% (standard deviation 3.2) conversion percentage was obtained for the calibration standards.

Having established the conditions for the reduction process, the study of aspiration volumes for both the reduced sample and colour reagent was carried out. The volume of colour reagent was set to obtain the maximum sensitivity within the expected dynamic range. The study of the volume of the sample to be aspirated from the column was made together with the study of the volume of sample sent to the column. A range from 60 to 115 μL was studied and, as the sensitivity increased up to the volume of 105 μL, this was the chosen value.

Concerning the determination of nitrate, two extra steps were included aiming for the preparation of the column for the next cycle and wash of the system. Firstly, the conditioner is aspirated through the column to remove residues of the previous sample (and leaving it filled with conditioner), and then (final step) the holding coil was washed preparing the system for the next cycle.

This way, the column was never left either empty or filled with water, therefore enabling to maintain a good percentage of conversion. Nevertheless, after some time that percentage decreased. When a percentage of conversion below 95% occurred, the cadmium was removed from the column and the procedures of washing, copperising and conditioning repeated before repacking into the column again.

3.3. Sequential determination of nitrite and nitrate.

Having optimised the conditions for both determinations, the aim was to accommodate both methods in the same manifold in order to perform the sequential determination of nitrite and nitrate in the same analytical cycle. For the simultaneous determination, during the stopping step in the nitrate determination, the determination of nitrite was carried out.

The possibility of having mixed standards of nitrite and nitrate was tested. As the results showed that there was no significant difference in the slopes (<2.8%) between the calibrations with single standards and mixed standards, mixed standards were used.

As two different flow cells were used in the combined arrangement, two ports of the selection valve were connected to the spectrophotometer. The optical path of these two cells were aligned in the cell compartment. As the two measurements, corresponding to each determination, are sequential there was no mutual signal interference. Firstly, the coloured product formed by nitrite reaction flows through the 2 cm flow cell. When the product of the nitrite + nitrate reaction is sent to the 1 cm flow cell, the 2 cm flow cell is filled with carrier (deionised water).

The possible salinity effect was assessed by comparing calibration curves using standard solutions with different salinities; these solutions were prepared by adding sodium chloride to the standards. The salinity values were adjusted to an intermediate and a maximum level of salinity in estuarine waters, 9 and 19 respectively. The calibration curve with pure standards was compared to the calibration curve with standards with salinity 9. The estimated slopes of both curves were not statistically different (assessed by the confidence intervals at 95%, which overlapped). The same was verified when the pure standards calibration curve was compared with the calibration curve with salinity 19, and for both nitrite and nitrate calibration curves.

A global linear regression was carried out using all data sets (a total of 116 experimental points) related to all calibration curves. The estimated linear model obtained was the following: $A = 5.06 \times 10^{-2} \pm 1.08 \times 10^{-3} [NO_2^-] - 7.42 \times 10^{-3} \pm 4.94 \times 10^{-3}$. The model parameters were similar to the ones estimated when only the pure standards data values were used (80 experimental points): $A = 5.06 \times 10^{-2} \pm 1.02 \times 10^{-3} [NO_2^-] - 1.24 \times 10^{-4} \pm 4.68 \times 10^{-3}$. This was also assessed by the magnitude of the confidence intervals of the slope and the intercept at 95% presented in the previous equations (*i.e.* ± half of the confidence interval at 95%). For the nitrate, the results obtained showed that calibration curves with different salinities were also identical. The estimated calibration curve was $A = 3.15 \times 10^{-3} \pm 6.46 \times 10^{-5} [NO_3^-] - 7.24 \times 10^{-2} \pm 1.02 \times 10^{-2}$ (global data with 116 data points) and $A = 3.17 \times 10^{-2} \pm 7.57 \times 10^{-5} [NO_3^-] - 8.38 \times 10^{-2} \pm 1.19 \times 10^{-2}$ (pure standards, 80 points).

As mentioned in Section 3.1, the quality of the regression was tested by residuals analysis (*i.e.* randomness and normality) and by the coefficient of determination (*i.e.* R^2, which was above 0.987 in all cases). Overall it can be concluded that the salt addition did not affect the calibration curves.

3.4. Features of the developed system

Some of the characteristics of the developed methodologies, like the dynamic concentration ranges, limits of detection (LOD) and quantification (LOQ) and repeatability (relative standard deviation, RSD) are summarized in Table 3.

The LOD and LOQ were calculated as the concentration corresponding to three and ten times the standard deviation of ten consecutive injection of deionised water, respectively, according to IUPAC recommendation.[15]

As mentioned in Section 3.1., linear regressions were assessed by residuals analysis (randomness, independence and normality verified) and the quality of the regression confirmed by the coefficient of determination.

The sample frequency was calculated based on the time spent per cycle. A complete analytical cycle took about 3.6 min for both nitrite and nitrate determination, including the reduction in the cadmium column, resulting in a sample throughput of 33 determinations per hour, including the two parameters. A complete analytical cycle takes into account not only the times in the protocol sequence but also the time required to change valve position and pump direction.

An analytical cycle corresponds to a sample consumption of 680 μL per determination of nitrite and nitrate, along with an overall reagent consumption of: 17 mg sulfanilamide; 1.7 mg

Table 3 Some features of the developed sequential injection system

Analyte	Dynamic concentration range	LOD	LOQ	% RSD, conc. ± standard deviation
Nitrite	0.50–8.00 μM	0.11 μM	0.36 μM	4.1%, 0.78 ± 0.04 μM (38.2 ± 1.6 μg L^{-1})
	(23–400 μg L^{-1})	(5.4 μg L^{-1})	(18 μg L^{-1})	1.1%, 5.88 ± 0.06μM (284 ± 3 μg L^{-1})
Nitrate	12.5–305 μM	3.7 μM	12.2 μM	3.1%, 54.2 ± 1.7μM (3.5 ± 0.1 mg L^{-1})
	(0.80–20 mg L^{-1})	(0.2 mg L^{-1})	(0.8 mg L^{-1})	0.8%, 304 ± 2μM (19.4 ± 0.1 mg L^{-1})

N1NED; 41 mg phosphoric acid, 181 μg EDTA, 9.0 mg ammonium chloride and an effluent production of about 6.8 mL.

The conversion percentage was assessed daily for two weeks resulting in an average of 101% (standard deviation of 2.1). A statistical test (t-test) was used to evaluate if that value was not significantly different from 100%,[15] which was proved as the calculated t-value was 1.346 with a correspondent critical value 3.163 for a 95% significance level.

3.5. Application to water samples

Water samples from the three different locations of the estuaries of the three rivers of the NW of Portugal were used to compare the developed methodology with the reference procedure.

The water samples were analysed by the developed sequential injection (SIA) system. For the nitrite determination, the samples were also analysed by the reference procedure[14] (Ref. Met.) and the results obtained are presented in Table 4.

To evaluate accuracy, a linear relationship between C_{SIA} (μM) and $C_{Ref.\ Met.}$ (μM) was established; the equation found was: $C_{SIA} = 0.971\ (\pm 0.0504) \times C_{Met.Ref.} + 0.0614\ (\pm 0.0900)$, where the values in parenthesis are 95% confidence limits. These figures show that the estimated slope and intercept do not differ statistically from values 1 and 0, respectively. Therefore, there is no evidence for systematic differences between the two sets of results.[15] As mentioned in Section 3.1., the linear regression was assessed by residuals analysis and the quality of the regression confirmed by the coefficient of determination.

The nitrate determination results from the reduction to nitrite prior to determination of total nitrite and subtraction of the nitrite value. For the nitrate determination, recovery studies were carried out using the estuarine waters, the results are presented in Table 5.

For spiking the samples, volumes of 100 μL and 200 μL of nitrate standard solution (10 mM) were added to 10 mL of sample. The calculation of the recovery percentage was made according to IUPAC.[16]

The SIA methodology provided recovery ratios with an average of 103% (standard deviation 6.2) and a statistical test (t-test) was used to evaluate if that mean recovery value did not significantly differ from 100%.[15] Results showed that for a 95% significance level the recovery values did not differ from 100% as the calculated t-value was 1.673 with a correspondent critical value 2.593, thus indicating the absence of multiplicative matrix interference.

After the validation of the developed methodology, the values of nitrite and nitrate of the tested estuaries were assessed. Samples were collected on two different dates for each estuary. The values of nitrite and nitrate as well as the sample characteristics according to the different rivers and locations are summarised in Table 6.

As expected, it was observed that the highest values of both anions were in the estuary of river Ave as it crosses a rural area. Overall, the lowest values were obtained for river Douro, being a dense urban area but with the higher discharge rate.

4. Conclusions

The developed sequential injection system proved to be very effective for the simultaneous determination of nitrite and nitrate

Table 4 Application of the developed system (SIA) to nitrite determination in estuarine water samples and comparison with the reference method (Ref. Met.); RD – relative deviation

River Estuary	Sample ID	pH (25 °C)	G/μS cm^{-1}	Salinity	Ref.Met./μM	SIA/μM	RD (%)
Douro	RD1	8.08	23300	15.2	0.470 ± 0.014	0.403 ± 0.020	−14.3
	RD2N	7.56	9230	5.8	0.791 ± 0.014	0.701 ± 0.084	−11.4
	RD2F	7.72	5480	3.3	0.446 ± 0.037	0.488 ± 0.042	9.3
	RD3F	8.67	6540	4.0	1.096 ± 0.024	1.144 ± 0.020	4.3
	RD3	9.17	1710	<2	0.739 ± 0.085	0.709 ± 0.030	−4.0
Cávado	RC1	7.23	7280	4.5	0.405 ± 0.145	0.403 ± 0.035	−0.6
	RC2F	6.97	2330	<2	1.353 ± 0.000	1.474 ± 0.012	9.0
	RC2N	7.11	2250	<2	1.072 ± 0.014	1.195 ± 0.012	11.5
	RC3F	7.11	695	<2	1.586 ± 0.028	1.601 ± 0.014	0.9
	RC3N	6.92	765	<2	1.707 ± 0.014	1.854 ± 0.023	8.6
Ave	RA3	8.38	607	<2	5.064 ± 0.000	4.908 ± 0.000	−3.1

Table 5 Application of the sequential injection system for nitrate determination in spiked estuarine water samples and respective recovery studies

				Initial			Added	Found			
Sample ID	pH (25 °C)	G/μS cm^{-1}	Salinity	Conc./μM	SD	RSD %	Conc./μM	Conc./μM	SD	RSD %	Recovery (%)
RD1	8.16	24300	16.5	57.5	1.1	2.0	100	163	1.8	1.1	105.5
				57.5	1.1	2.0	200	263	3.7	1.4	102.8
RD1F	8.18	24500	16.7	38.3	0.3	0.8	100	138	1	1.0	99.7
				38.3	0.3	0.8	200	236	13	5.7	98.9
RD2	8.37	23100	15.6	55.7	0.4	0.7	100	160	1.6	1.0	104.3
				55.7	0.4	0.7	200	256	3.6	1.4	100.1
RD2F	8.34	21700	14.6	44.1	1.8	4.1	100	150	3.9	2.6	105.9
				44.1	1.8	4.1	200	253	2.6	1.0	104.4
RD3	8.03	1087	< 2	64.7	0.1	0.2	100	158	2	1.1	93.9
				64.7	0.1	0.2	200	253	8	3.1	94.1
RD3F	8.12	938	< 2	59.1	1.7	2.9	100	158	14	8.9	98.9
				59.1	1.7	2.9	200	267	12	4.4	104.0

Table 6 Application to the NW Portugal estuaries

River Estuary	Date	Location	pH (20 °C)	G/μS cm^{-1}	Salinity	[NO_2^-]/μM ± SD	[NO_3^-]/μM ± SD
Douro	07-02-2008	1	7.88	17870	11.8	0.403 ± 0.020	14.4 ± 2.1
		2	7.56	9230	5.8	0.488 ± 0.084	58.7 ± 2.1
		3	8.49	2010	<2	0.709 ± 0.030	40.8 ± 2.0
	20-04-2008	1	8.04	14160	9.2	0.828 ± 0.011	70.9 ± 0.6
		2	7.72	5480	3.3	0.476 ± 0.011	71.9 ± 0.7
		3	8.67	6540	4.0	0.391 ± 0.020	71.4 ± 1.1
Cávado	10-02-2008	1	8.14	20500	13.7	0.663 ± 0.020	54.4 ± 0.5
		2	7.11	2250	<2	1.19 ± 0.01	65.4 ± 3.1
		3	6.92	765	<2	1.85 ± 0.02	111 ± 2
	06-05-2008	1	7.63	17860	11.8	0.333 ± 0.020	24.3 ± 0.5
		2	6.97	2330	<2	1.03 ± 0.01	87.9 ± 0.6
		3	7.11	695	<2	1.15 ± 0.02	76.9 ± 1.5
Ave	31-01-2008	3	8.83	334	<2	5.55 ± 0.04	211 ± 1
	05-05-2008	3	8.38	607	<2	3.35 ± 0.04	211 ± 1

in estuarine water samples. The versatility of sequential injection was explored for a biparametric determination with different sensitivity requirements using two different optical paths, achieved by connecting two flow cells to the selection valve. The simultaneous determination of nitrite and nitrate was accomplished with a single manifold which included the reduction process, through the copperised cadmium column. It should be emphasised that cadmium was kept inside the mini-column, with no contact with the operator. Additionally, as this mini-column only contained a small amount of cadmium, it is estimated that the cadmium consumption is about 50 times lower than the one used in the reference method.

The methodology was applied to natural samples, from three estuaries in NW Portugal, and the results proved its efficiency. The different composition of the samples did not interfere in the determination as the developed method proved to be quite robust for a wide salinity range, from <2 to 16.7, leaving the possibility for real time measurement.

When compared to previously reported colorimetric flow systems for these determinations, it presents the advantage of direct introduction of estuarine waters, with no need for prior treatments. Other advantages are a low reagent consumption and effluent production. Additionally, as this methodology provides analysis on real time, it could be used for early detection of pollution by fertilizers in freshwater streams and estuaries.

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